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COLLABORATIVE STUDY OF METHODS FOR ANALYSIS OF TOBACCO, NICOTINE, AND MOISTURE

BY

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NICOTINE AND MOISTURE

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A report is hereby presented of data obtained in the initial collaborative study of methods for determining nicotine and moisture in tobacco, conducted by an informal committee of tobacco research chemists.* The studies were designed to determine which of several methods described in the literature and in common use would prove suitable either in their present form or in some modified form as standard procedures for the analysis of tobacco for moisture and nicotine. The collaborators submitted not only their analytical results but also detailed descriptions of their methods. They were requested to send all the values obtained so that a statistical analysis of the data could be made, both to compare the different procedures (parts of the methods) and to show the effects of these procedures on the precision and accuracy of the method.

Two samples were sent to each tobacco analyst who had signified a willingness to participate in the studies. One was an acidified aqueous

* A committee on standardization of analytical methods for tobacco products, established informally by a group of tobacco research chemists representing government, university, and industrial organization, as a result of a tobacco research conference at the Eastern Regional Research Laboratory in October, 1947.

solution of nicotine and the other was Pennsylvania cigar leaf ground finer than 100 mesh. By using the liquid sample, the effect of variables inherent in moisture analysis and extraction of nicotine from tobacco plant tissue were largely eliminated; this allowed a better appraisal of the methods for nicotine.

NICOTINE ANALYSIS OF LIQUID SAMPLES

Results of analysis for nicotine in the liquid samples were reported by 17 collaborators. Ten analysts reported 41 values by the spectrophotometric method (4) and 17 analysts reported 86 values by the gravimetric method (2). Tables 1 and 2 list statistical summaries of the results. In these tables, n is the number of nicotine values reported by each collaborator, $\overline{X}$ is the mean of his data, and s is the standard deviation. The median value is the middle value obtained when all the data are arranged in ascending or descending order. The median value was used in these studies in lieu of a true value, because the true nicotine value is unknown and a median value is less affected by high or low results than a mean value would be.

TABLE 1.—*Statistical summary of nicotine values obtained by gravimetric analysis of liquid samples*

COLLAB. NO.	n	$\bar{X}$	s	$\bar{X}$-MEDIAN
		per cent		
0	6	0.99	0.000	+0.01
2	8	0.88	0.003	−0.10
3	4	1.00	0.002	+0.02
5	8	0.93	0.022	−0.05
6	6	1.01	0.015	+0.03
8	8	0.98	0.006	0.00
9	2	0.98	0.000	0.00
10	5	0.94	0.010	−0.04
11	4	0.99	0.000	+0.01
12	4	1.00	0.010	+0.02
13	4	0.99	0.000	+0.01
14	4	0.99	0.000	+0.01
16	3	0.97	0.000	−0.01
18	6	0.99	0.000	+0.01
19	4	0.98	0.002	0.00
20	8	0.98	0.010	0.00
21	2	1.00	0.010	+0.02
17	86			
Mean		0.98	0.005	0.01

Median = 0.99%
$s_{\bar{x}}$ = 0.032%

The mean of the $\overline{X}$'s for values by the gravimetric and spectrophotometric methods is 0.98 and 0.97, respectively. These values are almost identical with the median of a composite of all the values submitted. This was expected, since the average difference between the median and the average, $\overline{X}$, of each analyst's values is 0.01 for both methods. The average of the $\overline{X}$'s for both methods falls on the median for all

TABLE 2.—*Statistical summary of nicotine values obtained by spectrophotometric analysis of liquid samples*

COLLAB. NO.	n	$\bar{X}$	s	$\bar{X}$-MEDIAN
		per cent		
0	6	0.97	0.000	−0.01
2	6	0.77	0.054	−0.21
5	8	0.97	0.002	−0.01
10	3	0.97	0.002	−0.01
14	2	0.98	0.000	0.00
16	1	1.03	0.000	+0.05
18	2	0.97	0.000	−0.01
20	8	0.97	0.003	−0.01
21	2	0.97	0.028	−0.01
22	3	1.06	0.122	+0.08
10	41			
Mean		0.97	0.021	0.00

Median = 0.97%

$s_{\bar{x}}$ = 0.076%

the nicotine values. Student's t test was applied to determine if there is a significant difference between the means of the values of the two methods so that the one in closest agreement with the best measure of the true value (median value) can be established.

Since the calculated t, 0.518, is less than 2.060 t at the 95% level, there is no significant difference between the two methods. From these experiments it can be assumed that the two procedures studied for the analysis of "pure" nicotine in solution under the conditions specified are satisfactory as referee methods. Therefore, in the analysis of a plant material for nicotine, any apparent differences in the results submitted by the collaborators will be due either to differences in the methods of moisture analysis, in the procedures for recovering the nicotine from the plant, or in variations of the procedure for nicotine analysis.

MOISTURE ANALYSIS

The data on moisture, comprising 72 analyses furnished by the 18 collaborators, are summarized statistically in Table 3. These data have been treated in the same manner as the data on nicotine with n, $\overline{X}$, s, and $\overline{X}$ median. Here the median, 15.75%, and the mean of the $\bar{x}$'s are identical, and the mean of the s is only 0.071. The values for moisture represent a range of more than 2% and, as expected, the $s_{\bar{x}}$ is 0.793, showing that although the average precision for the analysts (s = 0.071) is acceptable, the precision for all the analysts is poor.

Based on these results, it was judged best to correlate the values for nicotine on an "as is" basis to eliminate any inherent errors in the moisture analyses. In doing so, it is assumed that there was less difference in the moisture of the ground leaf samples "as received" by the collaborators than in the moisture values reported. It can at least be assumed that there is no greater difference in the values reported on the "as is" basis than there is in the moisture values found.

NICOTINE ANALYSIS OF GROUND TOBACCO LEAVES

Nineteen analysts collaborated in the analysis of nicotine in ground tobacco leaves by the gravimetric silicotungstic acid method, and reported 97 values. The

statistical summary of these analyses is given in Table 4. The median value of 3.90 is derived from all the 97 analyses and not from the analysts' means ($\bar{X}$). However, the average of the analysts' means, 3.86%, compares favorably with the median of all values, 3.90%. The small average standard deviation of 0.064, as compared with 0.177 for $s_{\bar{x}}$'s indicates high precision for individual analysts but shows less precision when an inter-laboratory comparison is made. The low $\bar{X}$ median (−0.04) shows an average high accuracy. Nevertheless, there does exist a range of values of +0.26 to

TABLE 3.—*Statistical summary of moisture values of samples of ground tobacco leaves*

COLLAB. NO.	n	$\bar{X}$	s	$\bar{X}$-MEDIAN
		per cent		
0	8	16.00	0.041	+0.25
2	4	16.85	0.221	+1.10
3	3	15.20	0.000	−0.55
5	4	16.13	0.080	+0.38
6	1	15.91	0.000	+0.16
8	8	15.44	0.268	−0.31
9	2	15.77	0.014	+0.02
10	2	15.80	0.042	+0.05
11	2	15.45	0.000	−0.30
12	4	15.08	0.066	−0.67
13	2	15.88	0.000	+0.13
14	4	16.47	0.037	+0.72
16	6	15.69	0.116	−0.06
18	6	15.56	0.102	−0.19
19	3	16.62	0.050	+0.87
20	8	13.30	0.144	−2.45
21	2	16.73	0.036	+0.98
22	3	15.68	0.030	−0.07
18	72			
Mean		15.75	0.071	0.00

Median = 15.75%
$s_{\bar{x}}$ = 0.793%

−0.36, or a variation of 0.62%, in the nicotine found in this sample, which contained approximately 4% nicotine, indicating a possible error of 15% due to causes other than the determination of the nicotine in the distillate.

To compare the relative merits of the gravimetric and the spectrophotometric methods, the statistical analysis of the 45 values for nicotine by the spectrophotometric method submitted by 9 collaborators is presented in Table 5. The principal difference in the values obtained by the two methods is the $s_{\bar{x}}$, which is 0.498 for the spectrophotometric method and only 0.177 for the gravimetric. This difference indicates a wider spread between laboratories in the spectrophotometric results. Inspection shows that this spread is due to two laboratories, since one is 1.33% lower than the median, and the other 0.50% higher than the others. There is no apparent justification for deleting these two values, and statistically they cannot be dropped, yet if they were eliminated, the $s_{\bar{x}}$ value would be 0.129, which compares favorably with the $s_{\bar{x}}$ value of 0.177 for the gravimetric analyses.

TABLE 4.—*Statistical summary of nicotine values obtained by gravimetric analysis of ground tobacco leaves*

COLLAB. NO.	n	$\bar{X}$	s	$\bar{X}$-MEDIAN
		per cent		
0	8	3.91	0.022	+0.01
2	8	3.57	0.147	−0.33
3	2	4.16	0.000	+0.26
3	3	3.75	0.069	−0.15
5	6	3.88	0.021	−0.02
6	6	3.98	0.015	+0.08
8	8	4.00	0.046	+0.10
9	2	4.04	0.050	+0.14
10	5	3.88	0.050	−0.02
11	6	3.76	0.386	−0.14
12	4	3.79	0.030	−0.11
13	4	3.84	0.017	−0.06
14	4	3.60	0.050	−0.30
16	6	4.14	0.024	+0.24
18	6	3.91	0.037	+0.01
19	4	3.54	0.065	−0.36
20	8	3.75	0.017	−0.15
21	3	3.83	0.016	−0.07
22	4	3.98	0.159	+0.08
19	97			
Mean		3.86	0.064	−0.04

Median = 3.90%

$s_{\bar{x}}$ = 0.177%

TABLE 5.—*Statistical summary of nicotine values obtained by spectrophotometric analysis of ground tobacco leaves*

COLLAB. NO.	n	$\bar{X}$	s	$\bar{X}$-MEDIAN
		per cent		
0	8	3.92	0.024	+0.02
2	8	2.57	0.159	−1.33
5	6	3.93	0.068	+0.03
10	3	4.00	0.040	+0.10
14	4	3.64	0.050	−0.26
16	2	4.40	0.192	+0.50
18	3	3.93	0.129	+0.03
20	8	3.73	0.052	−0.17
21	3	3.81	0.040	−0.09
9	45			
Mean		3.77	0.084	−0.13

Median = 3.90%

$s_{\bar{x}}$ = 0.498%

EVALUATION OF VARIABLES IN METHODS FOR DETERMINATION OF MOISTURE AND NICOTINE

Since each collaborator described his modification of the method used for analyses of moisture and nicotine, it was possible to summarize all the variables in the procedures and correlate the data with one or the other of the many alternate variables of the procedures. Tables 6, 7, and 8 show the more important pairs of alternate variables for the methods of moisture, nicotine extractions, and nicotine analysis procedures, as well as the variable used by each collaborator.

By plotting the values obtained when either of a pair of variables was used, it is possible to show graphically which of the two variables contributes to greater accuracy. This is done by plotting the deviations of the $\overline{X}$ values from the median values, and indicating the average for each set of deviations. If the averages of the deviations for each of a pair of variables are essentially the same, it can be assumed that neither one of that pair has a significant effect on the accuracy of the results. If, on the other hand, the averages of the deviations are considerably different for any pair of variables, it is an indication that the differences in procedure have a significant effect on the accuracy. That procedure yielding an average deviation closest to the median value is to be preferred, since it tends to produce more accurate results. Plotted values for deviations from the median provide an easy method for appraising the effect of a variable, and this appraisal can be checked by application of the Student's t test (3) for unequal populations.

$$t = \overline{X}' \sqrt{\frac{n_a n_b (n_a + n_b - 2)}{(n_a + n_b)[(\overline{X}_a - \overline{X}_b)^2 + (\overline{X}_b - \overline{X}_b)^2]}}$$

where $\overline{X}' = \overline{X}_a - \overline{X}_b$; n_a and n_b are the number of values for a and b; X_a and X_b are the means of the individual values for the two groups, a and b, respectively; and $\overline{X}_a$ and $\overline{X}_b$ are the means of values of the two sets of values for a and b, respectively.

MOISTURE

In the analysis for moisture, the only variable found to be critical was the time of drying. The plot of deviations for values for heating times less than and greater than 3 hours is given in Fig. 1. The t test shows that the drying time is critical at the 90% level.

NICOTINE

A similar analysis was made of the effect of variations in procedure on the nicotine values of ground tobacco leaf. Plots of the deviation of the $\overline{X}$'s from the median nicotine value were made for the 18 different variations of the procedure. Plots are presented of only the 7 variations whose means were shown to be significantly different. In Fig. 2 is shown a comparison of the deviation from the median nicotine percentage of values obtained when the A.O.A.C. nicotine still was used and values obtained when other stills were used. The means of the analyst's $\overline{X}$'s indicate that the values obtained when the A.O.A.C. still was used are more accurate. This is confirmed by the t test, since the t at the 95% level, 2.064, is less than the calculated t, 3.542. The values obtained by the Griffith-Jeffreys still or its Willits-Connelly modification (1) give greater precision.

A comparison of the deviations of analysts' $\overline{X}$ values, obtained by using either NaOH or $Mg(OH)_2$ in the nicotine still pot, from the median nicotine percentage (Fig. 3), shows that the values obtained with NaOH are the most accurate. This is confirmed by the t test, since the t value found, 3.949, is larger than 2.086, t, at the 95% level. In this tobacco sample, although almost all the alkaloid was nicotine,

TABLE 6.—*Variables in methods used by the collaborators for the gravimetric determination of nicotine*[a]

	COMPARISON	COLLABORATOR NO.																		
		0	2	3	5	6	8	9	10	11	12	13	14	16	18	19	20	21	22	*n*
HCl added in precipitating nicotine	More than 1 ml	x					x	x		x		x		x				x		7
	Less than 1 ml		x	x					x		x		x			x	x			7
Volume silicotungstic acid per mg nicotine	More than 0.2 ml			x	x	x		x				x		x						6
	Less than 0.2 ml	x	x				x		x	x			x			x	x	x		9
Mg nicotine precipitated	More than 25 mg	x	x				x	x					x					x		6
	Less than 25 mg			x	x	x			x			x			x				x		7
Precipitate digested	Yes		x		x									x	x		x		x	6
	No	x		x		x	x	x	x	x	x	x	x			x		x		12
Crystallization	At room temp.	x		x		x	x	x	x	x	x	x	x	x		x		x		13
	Refrigerated		x		x										x		x		x	5
Filtering medium	Filter paper	x		x		x	x	x		x	x	x	x	x		x		x	x	13
	Other		x		x				x						x		x			5
Precipitate washed with H_2O	Yes						x										x			2
	No	x	x	x	x	x		x	x	x	x	x	x	x	x	x		x	x	16
Volume of acid wash solution	More than 100 ml			x		x	x	x		x				x	x	x		x		9
	Less than 100 ml	x	x		x				x		x	x	x				x		x	9
Ignition	Muffle furnace		x	x	x	x		x			x	x	x	x	x	x	x		x	13
	Meker burner	x					x			x								x		4
Ignition temperature	More than 800°C.	x		x		x		x				x	x					x		7
	Less than 800°C.		x		x					x	x			x	x	x	x			8
Time of ignition	More than 30 min	x	x	x	x	x		x				x				x	x		x	10
	Less than 30 min						x			x	x		x					x		5

[a] x=variable used by collaborator.

TABLE 7.—*Variables in methods used by the collaborators in the spectrophotometric determination of nicotine and determination of moisture*[a]

	COMPARISON	COLLABORATOR NO.																		
		0	2	3	5	6	8	9	10	11	12	13	14	16	18	19	20	21	22	*n*
Spectrophotometric determination of nicotine																				
No. of observations on spectrophotometer	More than 3	x			x								x				x	x	x	6
	Less than 3		x						x					x	x					4
No. of distillates	More than 3	x	x										x		x		x	x		6
	Less than 3				x									x					x	3
Moisture																				
Moisture dish	With cover	x		x	x	x			x	x			x			x	x	x	x	11
	Without cover		x					x			x	x		x	x					6
Drying temperature	More than 105°C.		x						x	x	x		x		x	x		x		8
	Less than 105°C.	x		x	x	x		x				x		x			x		x	9
Drying time	More than 3 hr.	x	x	x	x			x					x	x		x		x	x	10
	Less than 3 hr.								x		x	x			x		x			5
Sample size	More than 2.5 g	x		x		x		x				x			x			x		7
	Less than 2.5 g				x				x		x			x			x		x	6
Oven	Mechanical	x				x		x						x	x	x	x			8
	Gravity convection		x	x	x				x	x	x	x	x	x						9

[a] x = variable used by collaborator.

TABLE 8.—*Variables in methods used by the collaborators for nicotine extraction*[a]

	COMPARISON	COLLABORATOR NO.																		
		0	2	3	5	6	8	9	10	11	12	13	14	16	18	19	20	21	22	*n*
Sample size	More than 2 g	x		x		x	x	x			x	x		x	x			x		10
	Less than 2 g				x				x	x			x			x	x		x	7
Sampling	Weighing directly	x		x		x	x	x	x	x	x	x		x		x	x	x	x	14
	By difference		x		x								x		x					4
Stills	A.O.A.C.			x			x	x				x		x						5
	Others	x	x		x	x			x	x	x		x		x	x	x	x	x	13
Steam supply	House	x	x	x	x	x	x	x	x		x	x			x					11
	Lab. generated									x			x	x		x	x	x	x	7
Steam purified by scrubbing	Yes	x																		1
	No		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	17
Steam source	Tap H_2O	x	x				x		x	x	x	x			x	x		x		10
	Distilled H_2O												x	x			x		x	4
Alkali	NaOH			x	x	x	x	x	x	x				x		x			x	10
	MgO		x	x							x	x	x				x	x		7
	Others	x				x									x					3
Alkali per 100 ml of residue	More than 0.1 mole			x	x			x			x	x		x	x					7
	Less than 0.1 mole		x				x						x			x	x	x		6
Sodium chloride	Added			x	x		x		x					x						5
	Not added	x	x	x		x		x		x	x	x	x		x	x	x	x	x	14
Hydrochloric acid in receiver	More than 2 ml			x		x	x			x	x	x		x	x	x		x	x	11
	Less than 2 ml	x	x		x				x				x				x			6
Time of distillation	More than 45 min	x		x		x	x	x				x		x		x	x	x		10
	Less than 45 min		x		x				x	x	x		x		x				x	8

[a] x=variable used by collaborator.

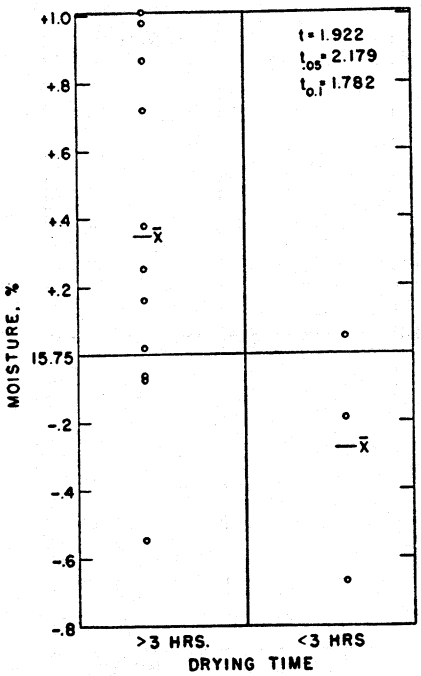

FIG. 1.—Deviations of the analysts' $\overline{X}$'s from the median value for moisture in ground tobacco leaf as influenced by drying time.

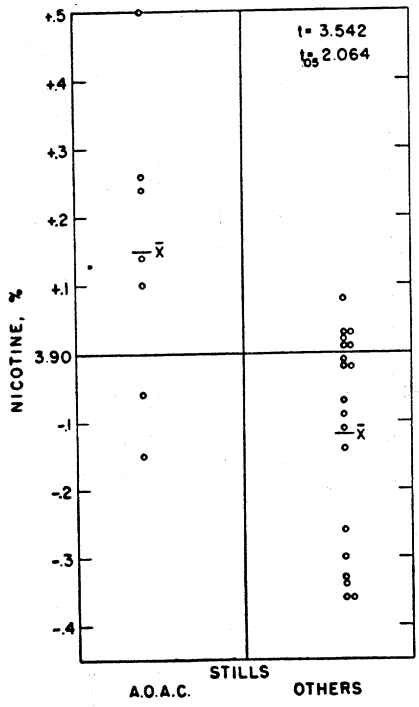

FIG. 2.—Deviations of the analysts' $\overline{X}$'s from the median value for nicotine in ground tobacco leaf as affected by the type of still.

the values obtained with MgO are, in all cases, lower than the median percentage of nicotine.

The concentration of alkali used in the nicotine still pot, measured in moles of alkali per 100 ml of residue left in the pot at the end of the distillation, has a significant effect on the accuracy of the results. Figure 4 shows that for concentrations of alkali greater than 0.1 mole, the accuracy is significantly better than that obtained for concentrations of less than 0.1 mole. This is confirmed by the t test, inasmuch as the calculated t, 4.678, is much larger than the t, 2.110, at the 95% level. This result indicates that unless care is taken to have an adequate concentration of alkali in the still, nicotine will be incompletely distilled.

The importance of the addition of sodium chloride to the still pot is demonstrated in Fig. 5. Here it is shown that the analyst's $\overline{X}$ values obtained when NaCl was used were much more accurate than those obtained when the NaCl was omitted. The t test shows that there is a significant difference, since the calculated t, 3.183, is larger than t, 2.074, at the 95% level. The fact that most of the low values were obtained when NaCl was not used indicates that the lower boiling temperatures of these still pot mixtures were not high enough to distill all the nicotine.

In these studies, the size of the sample taken for nicotine had a significant effect on the accuracy of the results obtained. Figure 6 shows that samples larger than 2 g gave more accurate values than were obtained when smaller samples were used.

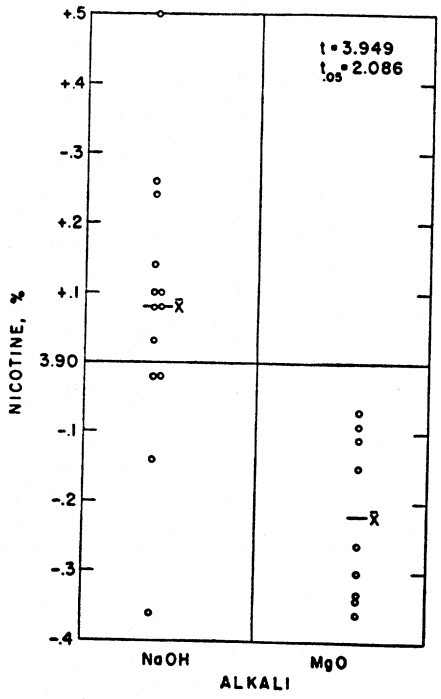

FIG. 3.—Deviations of the analysts' $\bar{X}$'s from the median value for nicotine in ground tobacco leaf as affected by the type of alkali.

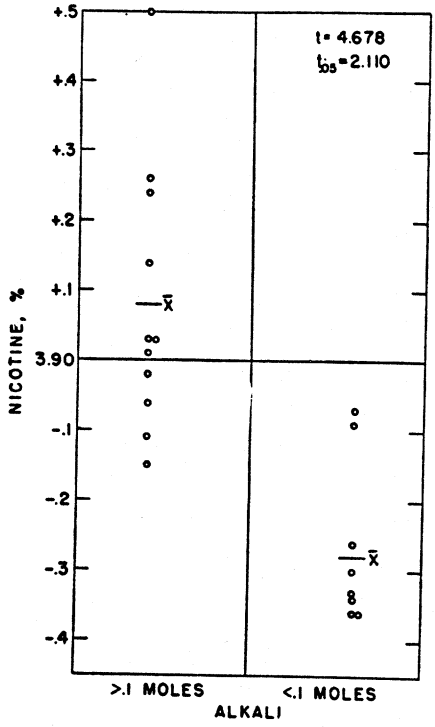

FIG. 4.—Deviations of the analysts' $\bar{X}$'s from the median value for nicotine in a ground tobacco leaf as affected by the concentration of alkali in residue.

This is confirmed by the t test, since the calculated t, 2.402, is much larger than t at the 95% level.

As expected, more accurate values were obtained when the liquid in the receiver was acid enough to convert *all* the nicotine to the less volatile salt. It can be seen from Fig. 7 that the values obtained when the receiving liquid contained more than 2 ml of acid were considerably higher and more accurate than when less acid was used. This difference is confirmed by the t test, since the calculated t, 2.281, is larger than t at the 95% level.

Another factor that appeared to have an important effect on the nicotine values was the amount of silicotungstic used to precipitate the nicotine. In Fig. 8 it is shown that the use of less than 0.2 ml of a 120 g/l solution of silicotungstic acid per mg of nicotine gave low and less accurate values. This is perhaps one of the most important of the factors that affect the accuracy of the results, since the calculated t is much larger than that for the 95% level.

DISCUSSION

In this collaborative study of methods for the determination of moisture and nicotine in tobacco, the statistical analysis of the data and the conclusions drawn can be interpreted only as indications of cause and effect on the values found. To draw more positive conclusions, it would have

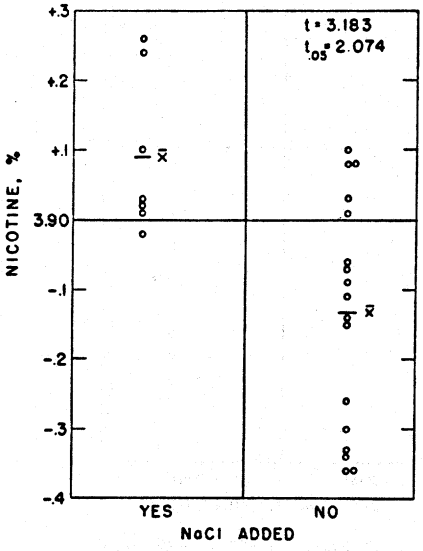

FIG. 5.—Deviations of the analysts' $\bar{X}$'s from the median value for nicotine in ground leaf tobacco as affected by the addition of salt to still pot.

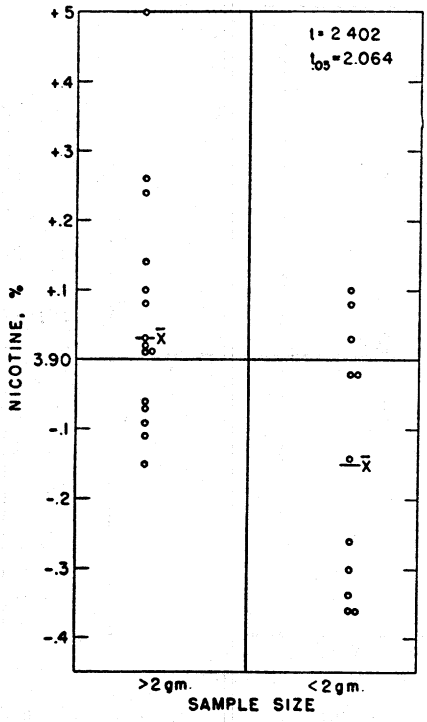

FIG. 6.—Deviations of the analysts' $\bar{X}$'s from the median value for nicotine in ground tobacco leaf as affected by the size of sample.

been necessary to adjust the data for each variable studied in terms of the data for the other variables. Because of the large number of variables considered and the relatively small number of analytical values for one variable, adjustment of the data to compensate for the effect of other variables would reduce the values of a particular series to such a small number that a statistical analysis would be valueless. The number of points in the figures do not necessarily agree with the number of collaborators who participated, as shown in Tables 6, 7, and 8 since often one collaborator submitted more than one set of data and in a few instances the values submitted were not included because they were received after the statistical analysis had been completed.

This study indicated that in the moisture analysis of ground tobacco leaf, conditions which do not influence the accuracy of the analyses are:

(1) Covered moisture dishes.
(2) Drying temperatures above or below 105°C.
(3) Sample size larger than or less than 2.5 g.

The time of drying had a slight effect on the moisture values. Drying

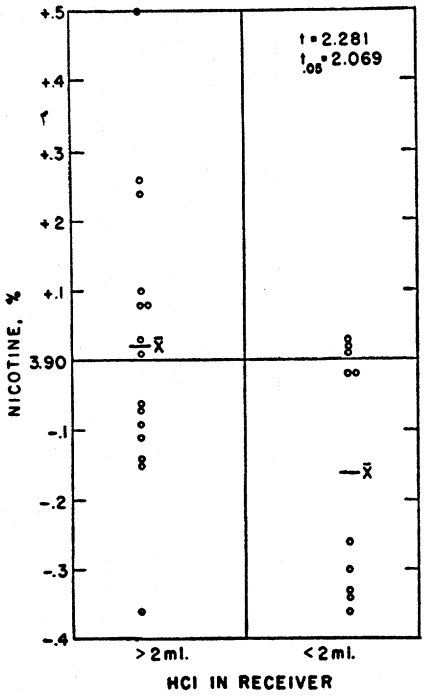

FIG. 7.—Deviations of the analysts' $\bar{X}$'s from the median value for nicotine in ground tobacco leaf as affected by the amount of hydrochloric acid in the receiver.

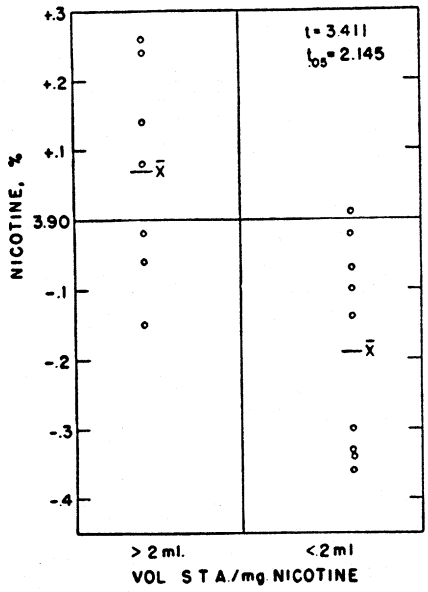

FIG. 8.—Deviations of the analysts' $\bar{X}$'s from the median value for nicotine in ground tobacco leaf as affected by the amount of silicotungstic acid used to precipitate the nicotine.

for more than 3 hours tended to give higher values.

In determinations of nicotine the A.O.A.C. silicotungstic acid gravimetric method and the spectrophotometric procedures gave equally precise and accurate values.

In the gravimetric procedure, the conditions that apparently do not affect the results within the ranges normally used are:

(1) Time of distillation.
(2) Test solutions that contain more or less than 25 mg of nicotine.
(3) Filtering medium.
(4) The use of more than or less than 100 ml of acid wash.
(5) Digestion of the precipitate on the steam bath.
(6) Ignition temperatures above or below 800°C.

Conditions that appear to have an effect on the results are:

(1) The sample should weigh more than 2 g.
(2) The use of sodium hydroxide in the still pot yields more nearly accurate results.
(3) More than 0.1 mole of alkali should be used in the still pot.

(4) Sodium chloride should be added to the distillation mixture.
(5) Until further data are obtained, the use of the A.O.A.C. still is preferred to all other stills. (Because of insufficient data, comparison between the A.O.A.C. and Griffith-Jeffrey stills could not be made.)
(6) The receiver should contain not less than 2 ml of concentrated hydrochloric acid.
(7) A minimum of 0.2 ml of silicotungstic acid should be used to precipitate each milligram of nicotine in the test solution.

SUMMARY

The data obtained suggest procedures that should be included in a standard method for nicotine analysis, but they should be confirmed by further study. The following conditions, however, have been fairly well defined:

(1) Care must be exercised in the time of drying (moisture analysis). This may be correlated to moisture of sample and temperature used.
(2) A sample larger than 2 grams should be used for nicotine analysis.
(3) Magnesium oxide will give lower values for total alkaloids as nicotine than sodium hydroxide.
(4) The still should contain more than 0.1 mole of alkali per 100 ml.
(5) The still pot mixture should contain sodium chloride.
(6) The receiver should contain more than 2 ml of concentrated hydrochloric acid.
(7) More than 0.2 ml of silicotungstic acid solution should be added per mg of nicotine.

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